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The p42/p44 MAP Kinase Pathway Prevents Apoptosis Induced by Anchorage and Serum Removal

Maude Le Gall,* Jean-Claude Chambard,* Jean-Philippe Breittmayer,† Dominique Grall,* Jacques Pouysségur,* and Ellen Van Obberghen-Schilling*‡

*Centre National de la Recherche Scientifique, Unite Mixte de Recherche, 6543, Centre A. Lacassagne, 06189 Nice Cedex 2, France; and †Institut National de la Santé et de la Recherche Médicale U 343, Hopital de l'Archet, 06202 Nice Cedex 3, France

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Anchorage removal like growth factor removal induces apoptosis. In the present study we have characterized signaling pathways that can prevent this cell death using a highly growth factor– and anchorage-dependent line of lung fibroblasts (CCL39). After anchorage removal from exponentially growing cells, annexin V-FITC labeling can be detected after 8 h. Apoptosis was confirmed by analysis of sub-G1 DNA content and Western blotting of the caspase substrate poly (ADP-ribose) polymerase. Growth factor withdrawal accelerates and potentiates suspension-induced cell death. Activation of Raf-1 kinase in suspension cultures of CCL39 or Madin–Darby canine kidney cells stably expressing an estrogen-inducible activated–Raf-1 construct (ΔRaf-1:ER) suppresses apoptosis induced by growth factor and/or anchorage removal. This protective effect appears to be mediated by the Raf, mitogen- or extracellular signal–regulated kinase kinase (MEK), and mitogen-activated protein kinase module because it is sensitive to pharmacological inhibition of MEK-1 and it can be mimicked by expression of constitutively active MEK-1 in CCL39 cells. Finally, apoptosis induced by disruption of the actin cytoskeleton with the Rho-directed toxin B (*Clostridium difficile*) is prevented by activation of the ΔRaf-1:ER chimeric construct. These findings highlight the ability of p42/p44 mitogen-activated protein kinase to generate survival signals that counteract cell death induced by loss of matrix contact, cytoskeletal integrity, and extracellular mitogenic factors.

INTRODUCTION

In addition to regulating growth, the extracellular matrix has been shown to control cell survival. It was reported a few years ago that normal cells deprived of matrix attachment undergo programmed cell death, termed anoikis (reviewed in Ruoslahti and Reed [1994]). Although first demonstrated in epithelial and endothelial cells (Frisch and Francis, 1994; Re *et al.*, 1994), anoikis has now been observed to varying extents in a number of cell types, including smooth muscle and neuronal cells as well as various fibroblastic cells (Ishizaki *et al.*, 1995; McGill *et al.*, 1997; Meredith and Schwartz, 1997; Valentinis *et al.*, 1998, 1999), and is reviewed in Meredith and Schwartz (1997). One biologically significant consequence of this phenomenon may be suppression of tumorogenesis, because the loss of anchorage dependence is one of the best correlates of tumoral growth in vivo. Indeed, expression of oncogenic Ras was found to prevent apoptosis after anchorage removal (Frisch and Francis, 1994; Re *et al.*, 1994).

‡ Corresponding author. E-mail address: vanobber@unice.fr.

The antiapoptotic action of oncogenic Ras has been documented in several cell types confronted with a variety of stresses including deregulated oncoprotein (e.g., c-Myc and E1A) expression, withdrawal of survival factors, or removal of extracellular matrix attachment, to name a few (reviewed in Downward [1998]). Attempts to identify the effector systems involved in this protective effect of Ras have indicated a role for the phosphatidylinositol 3′-kinase (PI3-K)[1]/Akt pathway (Marte and Downward, 1997; Downward, 1998; references therein). This is the case for apoptosis in Madin–

Abbreviations used: BSA, bovine serum albumin; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; *hid*, *head involution defective*; JNK, Jun-N-terminal kinase; MAP kinases, p42/p44 mitogen-activated protein kinases also known as ERK2 and ERK1, respectively; MDCK, Madin–Darby canine kidney; MEK-1, mitogen- or extracellular signal–regulated kinase kinase-1; PARP, poly (ADP-ribose) polymerase; PI3-K, phosphatidylinositol 3′-kinase; ΔRaf-1:ER, estrogen-inducible activated–Raf-1 construct; TUNEL, terminal deoxynucleotidyl transferase–mediated biotinylated UTP nick end labeling.

Darby canine kidney (MDCK) epithelial cells induced by detachment from the extracellular matrix. It was found that death in this system can be inhibited by expression of a constitutively activated form of Akt and that the extent of protection from apoptosis provided by Akt is comparable with that afforded by activated PI3-K or V12 Ras (Khwaja *et al.*, 1997).

Whereas studies addressing anchorage-dependent survival mechanisms have focused on PI3-K and Akt, less attention has been paid to the protective effect of the Ras/Raf/mitogen-activated protein (MAP) kinase pathway. We and others have observed that MAP kinase activation becomes compromised when cells are removed from the extracellular matrix (Lin *et al.*, 1997; Renshaw *et al.*, 1997; Le Gall *et al.*, 1998). This pathway was eliminated in MDCK cells on the basis of the findings that Raf-binding–defective mutants of Ras and Raf-CAAX, a plasma membrane–anchored form of Raf, were unable to block suspension-induced apoptosis (Khwaja *et al.*, 1997). Nonetheless, the MAP kinase pathway has been found to enhance cell survival in other systems and in response to various apoptotic stimuli, including death receptor-mediated activation (Holmstrom *et al.*, 1998; Yeh *et al.*, 1998) and survival factor removal (Xia *et al.*, 1995; Gardner and Johnson, 1996; Kinoshita *et al.*, 1997). Therefore, in the present study, we have specifically examined the anti-apoptotic effect of the Raf-1/MAP kinase pathway in response to anchorage removal using a system that allows selective activation of MAP kinase. In CCL39 or MDCK cells expressing an estrogen-inducible activated–Raf-1 construct (ΔRaf-1:ER), MAP kinase stimulation is rapid and persistent and does not involve transient expression of interfering or activating components of the pathway. Our findings clearly show that sustained activation of the Raf-1/MAP kinase pathway efficiently rescues CCL39 fibroblasts and MDCK epithelial cells from anoikis.

MATERIALS AND METHODS

Materials

Annexin V-fluorescein isothiocyanate (annexin V-FITC) was purchased from Boehringer Ingelheim Bioproducts (France). Estradiol was obtained from Sigma (St. Louis, MO), and anti-poly (ADP-ribose) polymerase (anti-PARP) antibody was purchased from Biomol (Plymouth Meeting, PA). The antibodies that specifically recognize the phosphorylated serine 473 of Akt and the anti-phospho-p42 and -p44 MAP kinases, as well as the specific mitogen- or extracellular signal–regulated kinase kinase-1 (MEK-1) inhibitor PD98059, were purchased from New England Biolabs (Beverly, MA). The rabbit polyclonal antibody (E1B3) that recognizes p42 and p44 MAP kinase was provided by Dr. F. McKenzie (Centre National de la Recherche Scientifique, Unite Mixte de Recherche 6543, Nice, France). Anti-Akt polyclonal antibody was a kind gift of Dr. B. Hemmings (Friedrich Mieschler Institute, Basel, Switzerland). The IL-1β–converting enzyme inhibitor VI Z-Val-Ala-D,L-Asp-fluoromethylketone and the PI3-K inhibitor LY294002 were from Alexis (Coger, France). Toxin B *(Clostridium difficile)*, purified as described previously (Von Eichel-Streiber *et al.*, 1987), was kindly provided by Dr. P. Boquet (Institut National de la Santé et de la Recherche Médicale U 452, Nice, France).

Cells and Culture Conditions

CCL39 Chinese hamster lung fibroblasts (American Type Culture Collection, Rockville, MD) and MEK SS/DD cells were maintained in DMEM (Life Technologies, Gaithersburg, MD) containing penicillin (50 U/ml) and streptomycin (50 μg/ml) and supplemented with 7.5% fetal calf serum (FCS). MEK SS/DD cells (a CCL39-derived subclone) express a constitutively active form of the MAP kinase kinase MEK-1 (Brunet *et al.*, 1994). MDCK cells were cultured in DMEM with 10% FCS, 1% nonessential amino acids, and 100 mM sodium pyruvate. CCL39–ΔRaf-1:ER cells (clones S18 and S19) (Lenormand *et al.*, 1996) and MDCK–ΔRaf-1:ER cells (clones Cl2 and Cl14) stably express a plasmid encoding ΔRaf-1:ER, an estradiol-regulated form of oncogenic Raf-1 kinase (provided by Dr. M. McMahon, University of California San Francisco Cancer Center, San Francisco, CA). ΔRaf-1:ER–expressing cells were cultivated in DMEM without phenol red and supplemented with penicillin, streptomycin, FCS, and G418 (400 μg/ml).

For experiments on cells in suspension, exponentially growing cells were detached from plates with trypsin (5 min, 37°C), and then the trypsin was blocked with soybean trypsin inhibitor (Sigma). After centrifugation, cells were resuspended in serum-free DMEM containing 1% bovine serum albumin (BSA) at 10^5 cells per ml and transferred to spinner flasks, as described previously (Le Gall *et al.*, 1998). Cells were stimulated, or not, in suspension with 10% FCS or estradiol for the indicated times.

Flow Cytometry

Annexin V-FITC labeling (10^6 cells per experimental condition) was performed as indicated by Bohringer Ingelheim Bioproducts. Briefly, cells were collected after different times in suspension or after detachment from plates with trypsin (5 min at 37°C followed by soybean trypsin inhibitor treatment). Cells were then rinsed with phosphate-buffered saline and incubated for 15 min in 100 μl of binding buffer (10 mM HEPES, pH 7.4; 140 mM NaCl; 5 mM $CaCl_2$) containing 2.5 μl of annexin V-FITC. Labeled cells were washed in binding buffer and analyzed on a FACStar (Becton Dickinson, Mountain View, CA). DNA content was determined on fixed cells stained with propidium iodide according to the protocol provided by Becton Dickinson.

Fluorescence Analyses

Suspended cells were labeled with annexin V-FITC, fixed in formalin (3.7%, 10 min at room temperature), and adsorbed on polylysine-coated slides. Terminal deoxynucleotidyl transferase–mediated biotinylated UTP nick end labeling (TUNEL) analysis was performed as described (Lassus *et al.*, 1996). Nuclei were stained for 15 min with 50 ng/ml 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Roche, Hertforshire, United Kingdom), and dead cells were revealed with propidium iodide. Polymerized actin was detected in cell monolayers using FITC-labeled phalloidin (Sigma). Slides were examined and photographed with a fluorescence-equipped photomicroscope (Diaphot; Nikon, Garden City, NY).

SDS-PAGE and Immunoblotting

For immunoblotting, cells were lysed in urea sample buffer (62.5 mM Tris-HCl, pH 6.8; 6 M urea; 10% glycerol; 2% SDS; 0.00125% bromophenol blue; 5% β-mercaptoethanol), sonicated 15 s, and incubated at 65°C for 15 min. This protocol, recommended by the manufacturer of the anti-PARP antibody (Biomol) for detection of PARP cleavage, was used for all immunoblot experiments. Proteins were separated by SDS-PAGE in 7.5% acrylamide gels and then transferred to Immobilon-P membranes (Millipore, Bedford, MA) and incubated with primary antibodies. After incubation with horseradish peroxidase–conjugated secondary antibody, immune complexes were detected with the enhanced chemiluminescence immunodetection system. Figures depict representative results from at least two independent experiments.

RESULTS

Anchorage Removal Induces Apoptosis in CCL39 Cells

Exponentially growing CCL39 cells were deprived of anchorage by placing them in suspension in spinner flasks, as described previously (Le Gall *et al.*, 1998). To reduce aggregation and the resulting cell–cell contacts that could activate intracellular-signaling systems, we supplemented the cell culture medium with 1% BSA.

In the absence of anchorage, a significant proportion of CCL39 cells die during an overnight incubation. Initially, cell death was assessed using a viability kit and by microscopic examination (our unpublished results). Thereafter, we used various methods to detect apoptosis in the suspended cell populations, including staining with annexin V-FITC that binds to phosphatidylserine present on the surface of apoptotic cells. Figure 1A shows annexin V-FITC–positive cells 24 h after detachment from the culture dish. At that time, DNA fragmentation can be detected by TUNEL analysis (Figure 1A).

As shown in Figure 1B, a peak corresponding to annexin V-FITC–positive cells can be readily detected by flow cytometry 8 h after cell detachment. By 24 h, nearly all of the cells analyzed in the experiment pictured here were positive. The appearance of annexin V-FITC–labeled cells coincides with the onset of a sub-G1 DNA peak (Figure 1C). Although there is evidence of DNA cleavage, including TUNEL labeling and the appearance of a sub-G1 DNA peak, we are unable to detect DNA ladder formation in these cells.

We next examined the cleavage of the PARP, a well-established substrate of caspase 3 and a marker of caspase cascade activation, in whole-cell extracts. A band of ~85 kDa corresponding to a cleavage product of PARP increases 24 h after anchorage removal, as shown in Figure 1D. Cleavage of PARP in suspended populations occurs with a slower time course than that of annexin V-FITC labeling suggesting that the membrane changes precede caspase activation. Altogether, these results demonstrate that CCL39 fibroblasts are susceptible to anoikis.

Serum Removal Potentiates the Induction of Apoptosis

Similar to anchorage removal, withdrawal of serum from the culture medium of attached cells also induces apoptosis in CCL39 cells, characterized by annexin V-FITC labeling, TUNEL analysis, and PARP cleavage (Figure 2, top). Apoptosis, as determined by PARP cleavage, seems to occur more rapidly after serum removal alone than after anchorage removal alone (compare Figures 1D and 2, top).

Although CCL39 cells undergo anoikis in the presence of serum, as shown above, they are much more sensitive to anchorage removal in its absence. As shown in Figure 2, bottom, in the absence of serum, PARP cleavage is markedly accelerated and potentiated, suggesting that mitogen withdrawal and anchorage removal trigger distinct cell death signals. We also observed that the extent of apoptosis depends on the confluence and growth rate of the cultures at the time of anchorage withdrawal. In contrast to epithelial cells, in which anoikis is more pronounced in confluent cultures (Frisch and Francis, 1994), maximal CCL39 cell death occurs in nonconfluent cultures of exponentially growing cells. Thus, in subsequent experiments, anoikis was maximally induced by placing exponentially growing cells in suspension in the absence of serum.

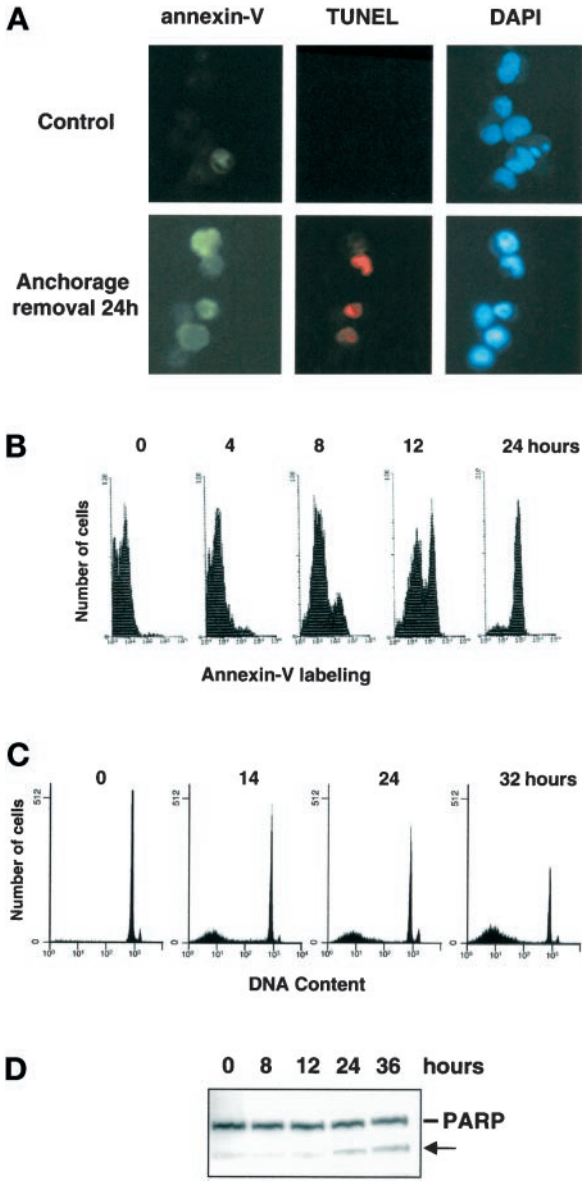

Figure 1. Anchorage removal induces apoptosis in CCL39 cells. Exponentially growing CCL39 cells were deprived of anchorage as described in MATERIALS AND METHODS. At the indicated times, cells were treated as follows. (A) Suspended cells were labeled with annexin V-FITC, fixed, and adsorbed on polylysine-coated slides before TUNEL analysis and nuclear staining with DAPI. Magnification: 630×. (B) Cells (10^6) were labeled with annexin V-FITC and analyzed by FACS. (C) Cells were fixed and incubated with propidium iodide, and DNA content was analyzed by FACS. (D) Immunoblot analysis of PARP was performed on total cell lysates. The arrow (here and in subsequent figures) indicates the position of the 85-kDa PARP cleavage product. Results shown are representative of at least two independent experiments.

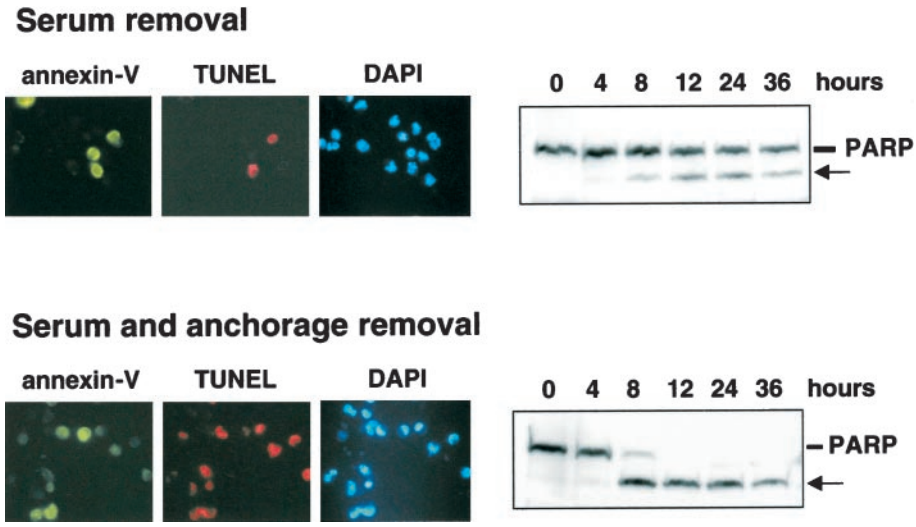

Figure 2. Serum removal potentiates induction of apoptosis. Exponentially growing CCL39 cells were deprived of serum (top) or anchorage and serum (bottom) as described in MATERIALS AND METHODS. At the indicated times, attached cells were trypsinized and treated as suspended cells. Annexin V-FITC labeling, TUNEL analyses, nuclei staining with DAPI, and immunoblot analysis of PARP were performed as described in Figure 1. Magnification: 400×.

We confirmed that the cleavage of PARP under these conditions is caspase dependent in CCL39 cells because it can be completely inhibited in the presence of Z-Val-Ala-D,L-Asp-fluoromethylketone, a wide-spectrum caspase inhibitor (Figure 3). After anchorage removal, we did not detect the appearance of a PARP cleavage product of ~50 kDa that results from necrotic cell death (Shah *et al.*, 1996). Thus, the analysis of PARP cleavage in this system is a reliable marker of apoptosis.

Selective Activation of the Raf-1/MAP Kinase Pathway

MAP kinase activity was found to be considerably lower in suspension cultures than in attached cells. Figure 4 shows a time course of MAP kinase phosphorylation after anchorage removal in the presence of serum, as detected by immunoblot analysis with anti-phospho-MAP kinase antibody.

To assess specifically the antiapoptotic action of the Ras/Raf/MAP kinase pathway after anchorage removal, without influence of PI3-K/Akt, we used a CCL39-derived cell line stably expressing a plasmid encoding an estradiol-regulated form of oncogenic Raf-1 kinase (ΔRaf-1:ER) (Lenormand *et al.*, 1996). Addition of estradiol to these cells induces a rapid and strong stimulation of p42/p44 MAP kinases, as shown in Figure 5. Activation persists as long as estradiol is present in the medium (i.e., Figure 5, left, 24 h). Stimulation of p42/p44 MAP kinase with estradiol is selective because no activating phosphorylation of Akt can be detected using anti-phospho-Akt antibody. In contrast, insulin at 10 μg/ml, which strongly and persistently stimulates Akt in monolayer cultures, presumably via the insulin-like growth factor-I receptor, has no detectable effect on p42/p44 MAP kinase activation (Figure 5, right).

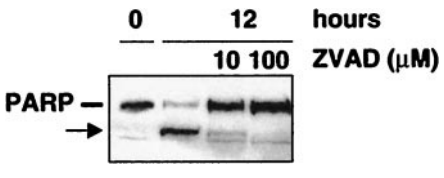

Figure 3. Z-Val-Ala-D,L-Asp-fluoromethylketone blocks apoptosis induced by anchorage and serum removal. Z-Val-Ala-D,L-Asp-fluoromethylketone (ZVAD) was added at the time of anchorage and serum removal. Immunoblot analysis of PARP was performed on total cell lysates from cells in suspension for the indicated times.

Conditional Activation of Raf-1/MAP Kinase Protects Cells from the Apoptosis Induced by Anchorage Removal

The addition of estradiol to CCL39–ΔRaf-1:ER cells in suspension markedly inhibits the apoptotic process triggered by anchorage and serum removal. The protective

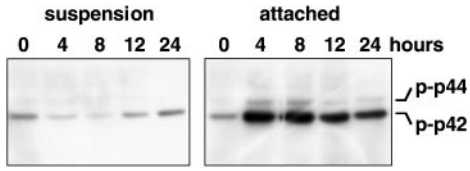

Figure 4. Comparison of MAP kinase activation in suspended and attached cells. Exponentially growing CCL39 cells were trypsinized, resuspended in serum-containing medium, and either placed in suspension (left), as described in MATERIALS AND METHODS, or replated in tissue culture dishes (right) for the indicated times. Immunoblot analysis of the active phosphorylated form of MAP kinase was performed on total cell lysates. p-p42, phospho-p42; p-p44, phospho-p44.

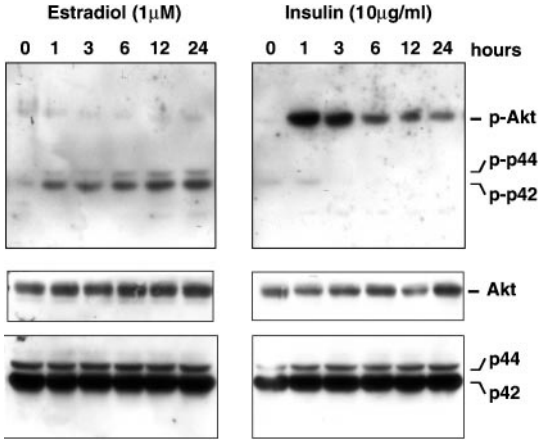

Figure 5. Raf activation in CCL39–ΔRaf-1:ER cells stimulates the MAP kinase pathway without activation of the PI3-K/Akt pathway. CCL39–ΔRaf-1:ER monolayers arrested in G0 by 18 h of serum removal were stimulated with estradiol or insulin for the indicated times. Western blot analyses were performed on whole-cell lysates using antibodies that recognize Akt or MAP kinase or their phosphorylated forms. p-Akt, phospho-Akt.

effect of Raf-1/MAP kinase can clearly be seen by microscopic examination of cells after an overnight incubation in suspension in the presence of estradiol (Figure 6A). Whereas estradiol-protected cells are round and refractile, cells in the nontreated cultures are shrunken and permeable to propidium iodide. Cleavage of PARP under these conditions is also markedly inhibited, for up to ≥24 h, in the presence of estradiol (Figure 6B). This protection correlates well with a dose-dependent activation of MAP kinase, as shown in Figure 6C. In medium containing 1% BSA, the maximally effective concentration of estradiol for MAP kinase activation is ~10-fold higher than that in the absence of BSA. It is also noteworthy that the anti-phospho-MAP kinase antibody used in our experiments recognizes the p42 isoform considerably better than the p44 isoform in CCL39–ΔRaf-1:ER cells. By gel mobility shift assays, we confirmed that both isoforms become activated by estradiol treatment under the same conditions (our unpublished results).

Protection Occurs Downstream of Raf-1 via the MAP Kinase Cascade

To determine whether the antiapoptotic effect of ΔRaf-1:ER could be attributed to activation of the MEK-1/MAP kinase module downstream of Raf-1 and not to another Raf-1–dependent system, we examined the effect of the MEK-1 inhibitor PD98059 on PARP cleavage. As shown in Figure 7A, this compound was able to reverse the protective effect of estradiol, suggesting a causal role for MAP kinase activation in the inhibition of PARP cleavage. Finally, we examined a CCL39-derived cell line (MEK SS/DD) that stably expresses a constitutively active form of the MAP kinase kinase, MEK-1. In this mutant, Raf-dependent regulatory phosphorylation sites serine 218 and 222 are both mutated to aspartic acids (Brunet *et al.*, 1994). These cells that display increased basal MAP kinase activity, as compared with parental cells, display a marked decrease in sensitivity to apoptosis induced by serum and anchorage removal, as determined by PARP cleavage (Figure 7B). Altogether, these results indicate that the antiapoptotic action of Raf-1 in response to anchorage and

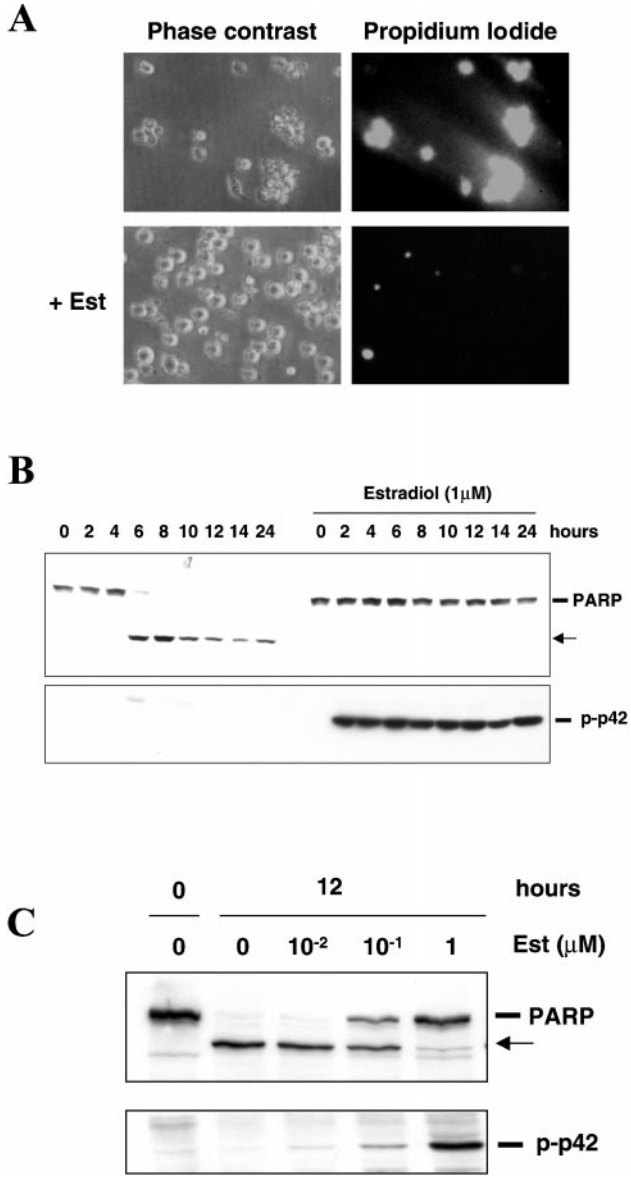

Figure 6. Inhibition of apoptosis after anchorage and serum withdrawal by Raf-1/MAP kinase activation. CCL39–ΔRaf-1:ER cells were deprived of anchorage and serum in the presence or absence of 1 μM estradiol (Est) for the indicated times. (A) Phase-contrast photomicrographs (magnification: 200×) of cells taken after 12 h (left) and propidium iodide staining (right) revealing dead, permeable cells. (B) Time course of PARP cleavage (top) and MAP kinase activation (bottom) after the detachment of cells in the presence or absence of estradiol. (C) Dose response of estradiol-induced inhibition of PARP cleavage (top) and activation of MAP kinase (bottom) after 12 h of anchorage and serum deprivation.

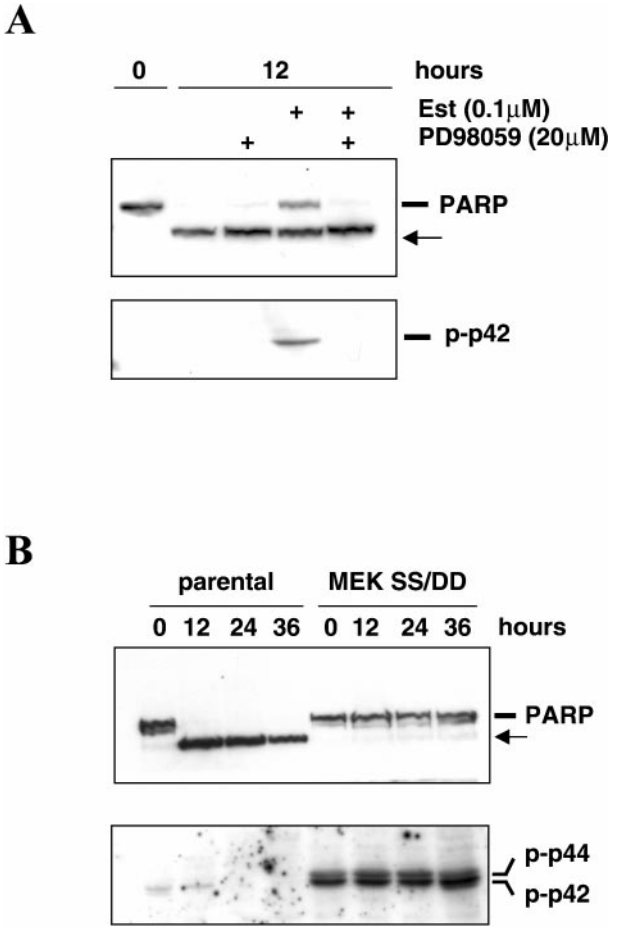

Figure 7. Effect of MEK-1 inhibition or stimulation on the cleavage of PARP. (A) Western blot analyses of PARP cleavage (top) and MAP kinase activation (bottom) were performed on total cell lysates from CCL39–ΔRaf-1:ER cells deprived of anchorage and serum for 12 h in the presence of 0.1 μM estradiol and 20 μM PD98059, as indicated (+). (B) Nontransfected cells (parental) or cells stably expressing constitutively active MEK-1 (MEK SS/DD) were deprived of anchorage and serum. At the indicated times, PARP cleavage (top) and MAP kinase activation (bottom) were analyzed by Western blotting.

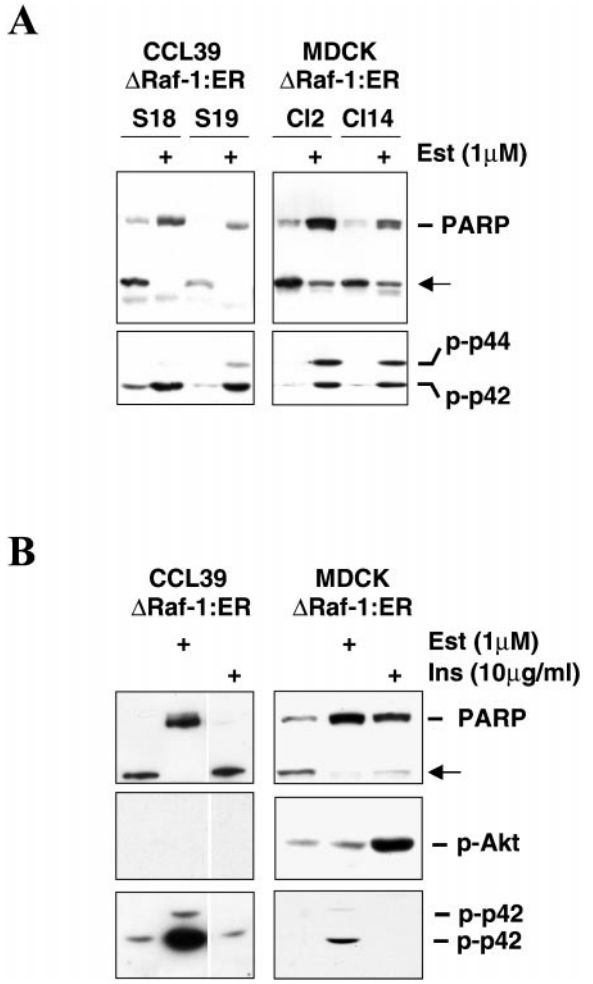

Figure 8. Inhibition of PARP cleavage in CCL39- or MDCK-derived clones expressing ΔRaf-1:ER. (A) Western blot analyses of PARP cleavage (top) and MAP kinase activation (bottom) were performed on total cell lysates from two independent clones expressing ΔRaf-1:ER after 10 h of anchorage and serum withdrawal in the presence (+) or absence of 1 μM estradiol. Clones S18 and S19 (left) were derived from CCL39; clones Cl2 and Cl14 (right) were derived from MDCK. (B) Western blot analyses of PARP cleavage (top), Akt activation (middle), and MAP kinase activation (bottom) were performed on total cell lysates from CCL39–ΔRaf-1:ER cells (clone S18) and MDCK–ΔRaf-1:ER clone 14 (Cl14) after 10 h of anchorage and serum withdrawal in the presence (+) or absence of 1 μM estradiol or 10 μg/ml insulin (Ins).

serum withdrawal is mediated by the MAP kinase–signaling pathway.

Antiapoptotic Action of the Raf/MAP Kinase Pathway in Other Cells

To verify that the protective effect of Raf-1 activation in CCL39–ΔRaf-1:ER cells was not restricted to the clone S18, described above, we examined PARP cleavage after anchorage removal in an independent CCL39-derived clone. This clone that homogeneously expresses ΔRaf-1:ER (referred to as S19) behaves similar to S18 in response to estradiol treatment, in terms of MAP kinase activation and PARP cleavage, as shown in Figure 8A, left. To extend these findings, we examined the ability of ΔRaf-1:ER to protect against anoikis in MDCK epithelial cells, an established model for the study of apoptosis induced by anchorage withdrawal. It is important to note that estradiol addition to both parental CCL39 and MDCK cells has no effect on MAP kinase activation or survival (our unpublished results). However, as shown in Figure 8A, right, cleavage of PARP is markedly reduced when estradiol is added to cultures of MDCK–ΔRaf-1:ER cells at the time of anchorage removal. It is noteworthy that inhibition of PARP cleavage is not complete in the two independent MDCK clones shown here because, in contrast to the CCL39-derived clones, ΔRaf-1:ER is not expressed in all of the cells (as determined by immunofluores-

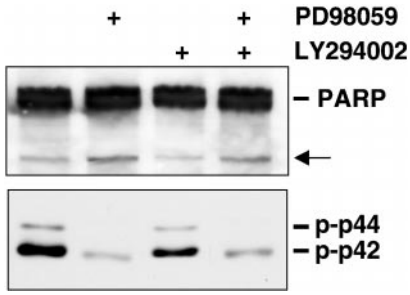

Figure 9. Effect of MAP kinase and PI3-K inhibition on PARP cleavage. Western blot analyses of PARP cleavage (top) and MAP kinase activation (bottom) were performed on total cell lysates from CCL39–ΔRaf-1:ER cells after 20 h of suspension culture in serum-supplemented medium in the presence (+) or absence of 20 μM PD98059 or 15 μM LY294002.

cence staining; our unpublished results). Nonetheless, protection correlates well with MAP kinase activation. Furthermore, estradiol does not activate Akt in suspended MDCK–ΔRaf-1:ER cells, similar to our observations in CCL39–ΔRaf-1:ER cells (Figure 8B).

In MDCK–ΔRaf-1:ER cells, protection from anoikis can be revealed in the presence of insulin, which activates Akt, as shown in Figure 8B, right. Interestingly, insulin is unable to protect CCL39-derived lines from undergoing apoptosis in suspension. In fact, we were unable to detect phosphorylated Akt in lysates from insulin-stimulated CCL39–ΔRaf-1:ER cells in suspension (Figure 8B, left). Altogether, these results confirm that the Raf/MAP kinase pathway represents an Akt-independent survival system.

Whereas the MAP kinase pathway clearly provides antiapoptotic signals, we did find that inhibition of this pathway is not sufficient to promote cell death. As shown in Figure 9, under conditions that allow detection of potentiating effects on cell death, such as anchorage removal in serum-containing medium for 20 h, inhibition of MAP kinase with PD98059 only slightly accentuated the cleavage of PARP in CCL39–ΔRaf-1:ER cells. No increase in the amount of cleaved PARP was detected in the presence of the PI3-K inhibitor LY294002, and this inhibitor did not further increase the effect of PD98059 alone (Figure 9).

Antiapoptotic Action of the MAP Kinase Pathway in Response to Rho Inhibition

The antiapoptotic potential of the conditionally active Raf-1 kinase with respect to apoptotic stimuli, other than anchorage or mitogen withdrawal, was examined in CCL39–ΔRaf-1:ER cells. Whereas CCL39-derived fibroblasts are quite resistant to several widely used inducers of apoptosis (e.g., anti-CD95 antibody, microtubule-disrupting agents, and tumor necrosis factor-α), we found that disruption of the actin cytoskeleton using a Rho-directed toxin had a strong apoptotic effect. Toxin B, a large *Clostridial* toxin produced by *C. difficile*, is a glucosyltransferase that inhibits RhoA, Rac1, and Cdc42 (Just *et al.*, 1995). This toxin has been found to disrupt actin filaments, to induce cell shrinkage, and to cause the appearance of neurite-like extensions in several different cell types (Siffert *et al.*, 1993; Just *et al.*, 1995; Chaves-Olarte *et al.*,

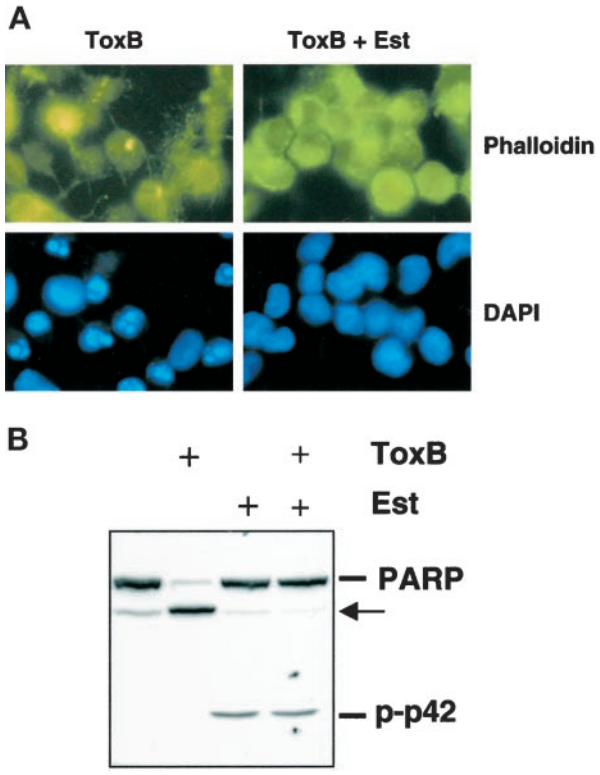

Figure 10. Antiapoptotic action of ΔRaf-1:ER after Rho inhibition. CCL39–ΔRaf-1:ER monolayers were treated with 60 pM toxin B (ToxB) in serum-free medium in the presence (+) or absence of 1 μM estradiol. (A) Polymerized actin was detected using phalloidin-FITC, and nuclei were labeled with DAPI. Magnification: 630×. (B) Western blot analyses of PARP cleavage (top) and MAP kinase activation (bottom) were performed on total cell lysates.

1997; Hippenstiel *et al.*, 1997). Apoptosis has also been observed after toxin B treatment of intestinal cells (Fiorentini *et al.*, 1998). As shown in Figure 10A, CCL39–ΔRaf-1:ER cells are sensitive to the toxin. After overnight treatment of cell monolayers with 60 pM toxin B, both disruption of the actin cytoskeleton and nuclear fragmentation can be readily observed. In addition, PARP cleavage in these cells is nearly complete (Figure 10B), indicating that inhibition of Rho family proteins leads to caspase activation. Interestingly, we found that persistent activation of MAP kinase with estradiol attenuated the cytoskeletal and nuclear effects of the toxin (Figure 10A). Furthermore, estradiol treatment of CCL39–ΔRaf-1:ER cells completely blocked cleavage of PARP (Figure 10B). Thus, activation of the Raf-1/MAP kinase pathway protects CCL39 cells from death induced by different apoptotic stimuli.

DISCUSSION

In the present study we have analyzed intracellular-signaling pathways that control anchorage-dependent survival of cells. We demonstrate here that 1) anchorage removal from growing CCL39-derived cells, like serum withdrawal, leads to apoptotic cell death and 2) activation of the Raf-1/MAP

kinase pathway provides a potent antiapoptotic signal in the absence of extracellular matrix support. In these fibroblasts, suppression of apoptosis by Raf-1 activation occurs without detectable activation of Akt, suggesting that the PI3-K/Akt pathway is not necessary for promoting survival of anchorage-deprived cells.

Our findings contrast those obtained using other cell systems that highlight the antiapoptotic function of PI3-K–activatable Akt kinase (reviewed in Franke *et al.* [1997] and Marte and Downward [1997]). Indeed, Akt has been suggested to promote cell survival by phosphorylation of BAD (Datta *et al.*, 1997; del Peso *et al.*, 1997), a proapoptotic member of the Bcl-2 family, by phosphorylation and inhibition of caspase-9 (Cardone *et al.*, 1998), or more recently by phosphorylation and cytoplasmic retention of the forkhead family transcription factor FKHRL1 (Brunet *et al.*, 1999). In the case of cell death triggered by extracellular matrix detachment, the group of Downward has shown in MDCK epithelial cells that cell death can be inhibited by expression of a constitutively activated form of Akt (Khwaja *et al.*, 1997). In their system, the contribution of Raf/MAP kinase to cell survival was excluded, on the basis of the observation that neither Raf-binding–defective mutants of Ras nor Raf-CAAX was able to block apoptosis (Khwaja *et al.*, 1997). In the present study, we observed significant protection from anoikis after activation of ΔRaf-1:ER in MDCK cells, suggesting that possible differences between ΔRaf-1:ER and Raf-CAAX may be caused by differences in the spatial distribution and/or intrinsic kinase activity of these two forms. In addition to the MAP kinase pathway, we observed significant protection from anoikis in MDCK cells via the PI3-K/Akt pathway stimulated by insulin. Thus, protection from anoikis can occur independently via activation of Raf-1/MAP kinase or PI3-K/Akt in these cells. In contrast, insulin failed to prevent apoptosis in suspended CCL39 cells. After anchorage removal, we were unable to detect Akt activation. In fact, Akt protein levels were found to decrease in these cells after prolonged anchorage and growth factor removal (our unpublished results). This loss of Akt responsiveness and of repression of the protein in cells undergoing anoikis is not unique to CCL39 cells; it was reported recently to occur in suspension cultures of human umbilical vein endothelial cells (Fujio and Walsh, 1999). Therefore, the relative contribution of the Raf-1/MAP kinase pathway and PI3-K/Akt pathway to cell survival may vary among cell types depending on the presence, or absence, of their respective signaling components.

How does anchorage removal trigger cell death? A number of scenarios could be envisioned. Cell death could be mediated by inactivation of one or more survival pathways. Indeed, it is clear that MAP kinase activation in response to serum is compromised when cells are placed in suspension (Lin *et al.*, 1997; Renshaw *et al.*, 1997; Le Gall *et al.*, 1998). However, we have shown here that the reduction in MAP kinase activity alone does not lead to cell death.

It is also possible that matrix removal could lead to induction of a death signal (via a receptor/ligand system or Bcl-2–regulated "apoptosome" pathway). To date we cannot exclude the involvement of signaling by cell-surface death receptors; however we have not been able to detect procaspase 8 cleavage or caspase 8 activity in lysates from suspended CCL39 cells (our unpublished results). We do observe cytochrome c release from the mitochondria of detached cells, suggesting that anchorage removal may trigger activation of a Bcl-2–regulated pathway involving Apaf-1 and procaspase 9 processing (our unpublished results). In MDCK epithelial cells, detachment from the matrix was found to activate Jun-N-terminal kinases (JNKs) (Frisch *et al.*, 1996), and this event has been suggested to play a determinant role in anoikis. In a subsequent report, activation of the JNK pathway failed to correlate with the induction of cell death in MDCK cells (Khwaja and Downward, 1997). We do not believe that this pathway contributes in a significant way to cell death in CCL39 cells because JNK does not become activated after anchorage removal (our unpublished results).

Although the signaling cues that trigger apoptosis after cell detachment from the matrix have not been fully elucidated, it is known that cell death can be inhibited by integrin engagement (Meredith *et al.*, 1993). Integrin ligation, like growth factor binding to receptors, leads to integrin clustering, assembly of focal adhesion complexes, and initiation of intracellular-signaling systems including the MAP kinase cascade (see Yamada and Geiger, 1997). Cell shape and spreading also appear to be required for integrin-dependent protection, at least in endothelial cells (Re *et al.*, 1994; Chen *et al.*, 1997), suggesting that integrin-activated pathways and the cytoskeleton cooperate for maintaining cell viability. This is consistent with our observation in CCL39-derived fibroblasts that inhibition of Rho family proteins and cytoskeletal disruption with toxin B result in apoptotic cell death.

The mechanism by which activated Raf-1 blocks apoptosis in CCL39–ΔRaf-1:ER cells is not yet clear. Certainly the high-intensity and persistent MAP kinase signal generated by estradiol in this these cells is not observed under normal physiological conditions. Nonetheless, this inducible system should prove to be extremely useful in unraveling the molecular mechanisms underlying this effect. Although a direct role for Raf-1 kinase in the protection of cells from apoptosis by cooperating with Bcl-2 has been proposed (Wang *et al.*, 1994, 1996), our studies indicate that protection from anchorage and/or growth factor removal occurs via activation of the downstream MAP kinases. In addition to the fact that the antiapoptotic activity of estradiol correlates well with activation of the MAP kinases, constitutively active MEK-1–expressing cells are clearly protected from apoptosis. Furthermore, pharmacological depletion of activated MEK-1 with PD98059 reverses ΔRaf-1:ER–mediated inhibition of PARP cleavage in suspended cells.

How does MAP kinase promote survival? What are the targets of MAP kinases involved in protection from cell death? These questions may be easier to resolve after we understand how anchorage removal induces apoptosis. The regulation of survival by MAP kinase could be controlled by the activation of antiapoptotic pathways, such as the induction and/or activation of either Bcl-2 or inhibitor of apoptosis (IAP) family members, or by the inhibition of proapoptotic pathways. In CCL39 cells, we have not been able to detect Bcl-2 expression; however we have observed that MAP kinase activation does not increase Bcl-XL expression (our unpublished results). Recently, Yeh *et al.* (1998) demonstrated that activation of MEK-1 leads to the expression of FLIP, a specific FADD inhibitor. This observation could

explain the protective effect of the MAP kinase pathway observed in the case of Fas-induced apoptosis in T cells (Holmstrom *et al.*, 1998). However, we do not believe that death receptor activation plays a major role in the apoptosis induced by anchorage removal, as mentioned above.

Recently, compelling genetic evidence from *Drosophila* supports a key role for the Ras/MAP kinase pathway in the protection against apoptosis by inhibition of the proapoptotic activity of the *head involution defective (hid)* gene (Bergmann *et al.*, 1998; Kurada and White, 1998). In the fly, deregulation by MAP kinase was observed at the transcriptional level resulting in the downregulation of *hid* expression and posttranslationally by phosphorylation and inactivation of the *hid* gene product. Thus far, no *hid*-like gene or functional equivalent of Hid has been identified in mammalian cells. However, it is likely that MAP kinase regulates a set of genes and/or proteins in mammalian cells that can act cooperatively to suppress apoptosis under conditions of appropriate integrin and growth factor receptor ligation. Our results suggest that MAP kinase may be required to link an integrin-mediated signaling event to cell survival. After integrin signaling is perturbed or mitogen/survival factors become limited, MAP kinase activity decreases, and cell death signals prevail. An understanding of these proapoptotic- and antiapoptotic-signaling pathways controlled by extracellular signals should provide valuable clues from which therapeutic approaches can be devised to block tumor cells from escaping these controls.

Note. While our manuscript was under evaluation, Erhardt *et al.* (1999) reported that overexpression of B-Raf in Rat-1 fibroblasts blocks apoptosis induced by serum deprivation without interfering with the release of cytochrome c from mitochondria, indicating that the Raf/MAP kinase pathway can inhibit apoptosis at the level of caspase activation.

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